

Molecular Cloning of Invasion Plasmid Antigen (*ipa*) Genes from *Shigella flexneri*: Analysis of *ipa* Gene Products and Genetic Mapping

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Tn5-tagged invasion plasmid DNA (pWR110) from *Shigella flexneri* serotype 5 (strain M90T) was cloned into the expression vector λ gt11. Recombinant phage (λ gt11Sf) expressing pWR110-encoded polypeptide antigens were identified by using rabbit antisera directed against *S. flexneri* M90T invasion plasmid antigens. Antigens encoded by λ gt11Sf recombinant phage were characterized by reacting affinity-purified antibodies, eluted from nitrocellulose-bound plaques of λ gt11Sf recombinants, with virulent, wild-type *S. flexneri* M90T polypeptides in Western blot analyses. λ gt11Sf clones directing the synthesis of complete, truncated, and β -galactosidase fusion versions of three previously identified outer membrane polypeptides (57-, 43-, and 39-kilodalton [kDa] antigens) were isolated. A fourth polypeptide, similar in size to the 57-kDa antigen (ca. 58 kDa) but unrelated as determined by DNA homology and serological measurements, was also identified. Southern blot analysis of *S. flexneri* M90T invasion plasmid DNA hybridized with λ gt11Sf insert DNA probes was used to construct a map of invasion plasmid antigen genes (*ipa*) corresponding to the 57-kDa (*ipaB*), 43-kDa (*ipaC*), and 39-kDa (*ipaD*) polypeptides. Genes *ipaB*, *ipaC* and *ipaD* mapped to contiguous 4.6-kilobase (kb) and 1.0-kb *Hind*III fragments contained within a larger (23-kb) *Bam*HI fragment. The *ipaH* gene, which encodes the synthesis of the 58-kDa polypeptide, did not map in or near the *ipaBCD* gene cluster, suggesting a distinct location of *ipaH* on the invasion plasmid.

The complex pathology of the dysenteric syndrome, caused by *Shigella* spp. and enteroinvasive *Escherichia coli*, is reflected in the diversity of genetic components controlling the virulence of these organisms. Both chromosomal and extrachromosomal loci that are essential for the expression of the virulent phenotype have been identified (21, 22; reviewed in reference 10). One aspect of this phenotype, the invasion of colonic epithelial cells, has its genetic components located on a large 120- to 140-megadalton (MDa) nonconjugative plasmid found in all *Shigella* and enteroinvasive *E. coli* strains (11, 23, 24). Loss of the plasmid is accompanied by loss of the invasive phenotype, as measured by in vitro infection of cultured mammalian cells, and by the inability of spontaneously cured shigellae to elicit keratoconjunctivitis (i.e., the Sereny reaction) in guinea pigs (21, 23, 24, 27). Reintroduction of the invasion plasmid into a plasmid-free avirulent *Shigella* strain restores the invasive phenotype (23, 24, 30).

A 37-kilobase (kb) region of the *S. flexneri* serotype 5 invasion plasmid cloned into the cosmid vector pJB8 restores the HeLa cell invasiveness of plasmid-cured *Shigella* spp. but does not restore the ability to cause a positive Sereny reaction (13). At least eight polypeptides, ranging in size from 12 to 140 kDa, have been identified as unique products of the invasion plasmid (7, 8). Four of these eight polypeptides, designated a (78 kDa), b (57 kDa), c (43 kDa), and d (39 kDa), are synthesized from the cloned 37-kb fragment (13). Polypeptides a, b, c, and d are coordinately expressed outer membrane polypeptides whose synthesis is thermoregulated, being repressed at 30°C, a nonpermissive

temperature for the invasive phenotype (13, 14). These polypeptides, plus an additional 140-kDa outer membrane protein, are also important immunogens, since convalescent-stage sera from infected humans and monkeys contain significant titers of antibodies recognizing these antigens (17). The association of plasmid-encoded polypeptide antigens with the invasive phenotype, as well as their ability to act as potent immunogens, suggests that the 140-, 78-, 57-, 43-, and 39-kDa antigens may be involved in eliciting a protective immune response in primates and humans. Characterization of these invasion plasmid antigen (*ipa*) genes will be an important step in the development of an effective dysentery vaccine.

In this study, we used the λ gt11 expression vector (29, 32, 33) to clone *ipa* genes from a Tn5-tagged derivative (pWR110) of the *S. flexneri* M90T 140-MDa invasion plasmid. Clones synthesizing the b (57 kDa), c (43 kDa), and d (39 kDa) antigens were isolated, and the corresponding genes were named *ipaB*, *ipaC*, and *ipaD*, respectively. A fourth gene, *ipaH*, was also defined, whose product is a protein similar in molecular mass (58 kDa) to but antigenically distinct from the *ipaB* gene product. Recombinants expressing complete, truncated, and β -galactosidase fusion versions of each *ipa* gene product were isolated. A genetic map of the *ipaBCD* gene cluster was constructed by using the cloned DNA fragments as probes.

MATERIALS AND METHODS

Construction of the λ gt11 expression library from pWR110 DNA. Tn5-tagged invasion plasmid pWR110 DNA was isolated from *S. flexneri* serotype 5 strain M90T by the procedure of Cassie et al. (3). M90T(pWR110) cells expressed the

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invasive phenotype as determined by positive reactions in both the HeLa cell invasion and Sereny assays (6, 24, 27). M90T(pWR110) synthesized four of five immunogenic outer membrane proteins that have been correlated with the invasive phenotype (7, 17) but did not synthesize detectable quantities of the 78-kDa protein, suggesting that this protein may not be an essential component of the invasive phenotype. pWR110 DNA was purified by centrifugation through a cesium chloride-ethidium bromide density gradient, and 50 µg of the DNA was partially digested with a mixture of six blunt-end-cutting restriction endonucleases. Six units each of *AatI*, *AluI*, *DraI*, *RsaI*, *EcoRV*, and *PvuII* were combined, diluted 1:10 in reaction buffer, and used to generate pWR110 insert fragments of approximately 0.4 to 6.5 kb. *EcoRI* sites on the insert DNA were methylated with *EcoRI* methylase, and phosphorylated *EcoRI* linkers (Pharmacia, Inc., Piscataway, N.J.) of three different lengths (8-mer, 10-mer, and 12-mer) were then blunt-end ligated to the insert DNA by using T4 DNA ligase supplemented with T4 RNA ligase and 1 mM spermidine. The ligated material was cleaved with an excess of *EcoRI* and separated from unligated linkers by passage of the mixture over a Sepharose 4CLB (Pharmacia) column. Eluate containing the insert DNA was subjected to a second excess *EcoRI* digestion followed by Sepharose 4CLB separation. The final insert DNA eluate was phenol extracted, precipitated with ethanol, suspended in ligation buffer, and ligated to *EcoRI*-cleaved λ gt11 arms (Promega Biotec, Madison, Wis.). The resulting recombinant phage were packaged into λ phage heads by using an in vitro packaging system (Promega Biotec) and were plated on *E. coli* Y1090 cells (Δ lacU169 *proA*⁺ Δ lon *araD139 rpsL supF trpC::Tn10 hsdR hsdM*⁺ *lacI*^r) (32) for screening. Recombinant phage produced colorless plaques on agar containing 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (X-gal). Restriction endonucleases, *EcoRI* methylase, T4 DNA ligase, and T4 RNA ligase were purchased from New England BioLabs, Inc., Beverly, Mass.

Screening the λ gt11 expression library and isolation of Y1089:: λ gt11Sf1 lysogens. Recombinant λ gt11 phage directing the synthesis of pWR110-encoded antigens were identified by using rabbit antisera specific for M90T invasion plasmid antigens b (57 kDa), c (43 kDa), and d (39 kDa), prepared as previously described (7.) Screening antisera were absorbed with whole-cell and membrane fractions of M90T-A2, an avirulent, invasion plasmid-cured derivative of M90T (24) and Y1090, the *E. coli* plating strain for recombinant λ gt11 phage. Membrane fractions were prepared by treating sonicated cells with Triton X-100. After absorption of the antisera, the membrane fractions were removed by centrifugation at $100,000 \times g$ for 1 h. A final absorption with λ gt11 phage particles (10^9 phage per ml of antiserum) was done before the antisera were used to screen the λ gt11 recombinants. Western blot analysis with the absorbed antisera against whole-cell lysates of M90T, M90T-A2, and Y1090 showed that the antisera clearly recognized invasion plasmid polypeptides of M90T but had minimal reactivity against M90T-A2 or Y1090 polypeptides.

The λ gt11 expression library was screened for antigen production essentially as described by Young and Davis (33). Recombinant phage (500 PFU/15-cm plate) were plated onto a lawn of Y1090 cells and transferred to isopropyl- β -D-thiogalactopyranoside (IPTG)-saturated (10 mM) nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) after the phage plaques became visible in 3 to 5 h. The nitrocellulose filters were blocked with a Tris-buffered saline solution (TBS; 10 mM Tris hydrochloride, 150 mM NaCl [pH

7.2]) containing 2% (wt/vol) casein. Screening antisera, diluted 1:200 in casein filler, were incubated with the nitrocellulose filters for 2 h at 25°C, the excess unbound antibody was washed off, and the filters were incubated with staphylococcal protein A (Pharmacia) labeled with ¹²⁵I, as previously described (7). Positive plaques (designated λ gt11Sf1), detected by autoradiography of the reacted filters, were isolated and purified three times by successive platings and screenings on Y1090 cells. Finally, λ gt11Sf1 recombinants were amplified to high titer and used to make lysogens in *E. coli* Y1089 cells (Δ lacU169 *proA*⁺ Δ lon *araD139 strA hflA* chr::Tn10 *hsdR hsdM*⁺ *lacI*^r) at multiplicities of infection of 5 to 50 (32).

λ gt11Sf1 phage plaques were isolated on Y1090 lawns grown at 37°C on L-agar plates containing 100 µg of ampicillin per ml. Y1089:: λ gt11Sf1 lysogens were similarly grown on L agar containing 100 µg ampicillin per ml at 32°C. IPTG and casein were purchased from Sigma Chemical Co., St. Louis, Mo.

Characterization of invasion plasmid antigens in Y1089:: λ gt11Sf1 lysogens. Y1089:: λ gt11Sf1 lysogens were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as follows. A single colony of the lysogen to be examined was inoculated into 20 ml of L broth and incubated with strong aeration of 32°C. Optical density measurements of the culture were monitored at 600 nm until an A_{600} of 0.4 to 0.5 was reached; the culture was then quickly shifted to 42°C and incubated for 25 min with vigorous aeration. IPTG (final concentration, 10 mM) was added to 10 ml of the temperature-induced culture; the untreated and IPTG-treated aliquots were then incubated at 37°C for 60 to 90 min. IPTG-induced and uninduced lysogens were collected by centrifugation, and the cell pellets were suspended in 0.5 ml of electrophoresis sample buffer (0.05 M phosphate buffer [pH 6.8], 2% SDS, 5% 2-mercaptoethanol 12% glycerol, 0.01% bromophenol blue, 0.005 mM phenylmethylsulfonyl fluoride) and heated in boiling water for 3 min. The gels consisted of 9% acrylamide cross-linked with *N,N'*-diallyltartardiamide. After electrophoresis, the components were electroblotted to nitrocellulose (2). Proteins of Y1089:: λ gt11Sf1 lysogens that reacted with the plasmid-specific screening antisera were identified by Western blot analysis, performed as previously described (2, 7, 17).

Affinity purification of antibodies (antigen-selected antibody) and identification of λ gt11Sf1 clones. Antibodies reactive with invasion plasmid antigen epitopes cloned in λ gt11Sf1 recombinants were affinity purified from the polyvalent rabbit screening antisera as described by Lyon et al. (12). Plaque-purified λ gt11Sf1 phage were plated onto a lawn of Y1090 cells such that 3×10^4 to 5×10^4 plaques would develop on a 150-mm petri dish. After the plaques became evident (3 to 5 h at 42°C), a nitrocellulose filter (diameter, 139mm), saturated with 10mM IPTG and blotted dry, was placed on the recombinant phage plaques. The filter was incubated on the plate at 37°C for 3 to 12 h, removed, washed twice in TBS for 5 min, and blocked with 2% casein in TBS. Rabbit antisera, diluted 100-fold, were then incubated with the filter for 3 h at room temperature. Unbound antibody was decanted, and nonspecifically bound antibody was removed by consecutive 10-min washes in TBS (once), TBS plus 0.05% Triton X-100 (twice), TBS (once), and saline (once). Antibodies bound to λ gt11Sf1 antigens were eluted by washing the filters with 10 ml of 0.2 M glycine-0.15 M NaCl (pH 2.8) buffer and then neutralizing them to pH 7.0 with Tris-base (8mg/ml). Eluate containing antigen-selected anti-

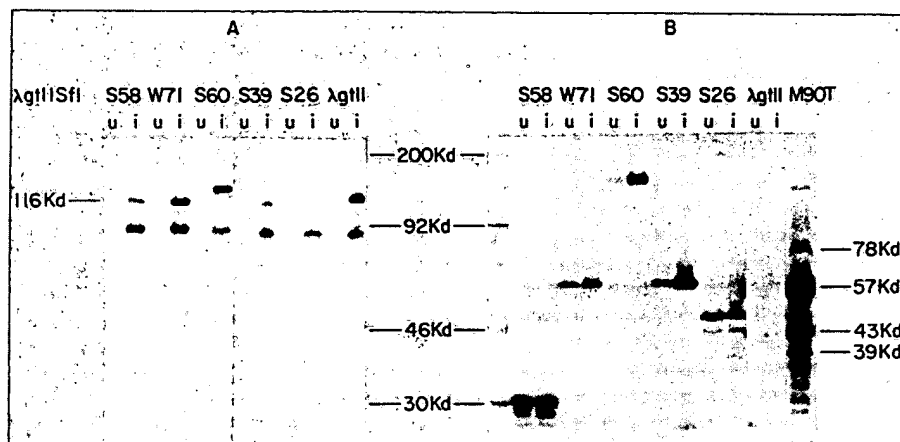


FIG. 1. Western blot of Y1089::λgt11SfI lysogens probed with anti-β-galactosidase antibody (A) and pWR110 antigen-specific rabbit antisera (B). Bound antibody was detected with 125 I-labeled staphylococcal protein A. Y1089::λgt11SfI lysogens (clone number indicated across the top of the panel) were heat induced for 25 min at 42°C and then split into aliquots containing 10 mM IPTG (i) or no IPTG (u). After incubation for 1 h at 37°C, cell lysates for SDS-PAGE were prepared as described in the text. A 14 C-labeled polypeptide size standard is included in panel B along with a virulent M90T control, with the positions of the M90T 78-, 57-, 43-, and 39-kDa polypeptides indicated to the right of panel B.

body was diluted with 2% casein and used in Western blots against whole-cell lysates of M90T to identify the antigen(s) cloned in a given λgt11SfI phage recombinant.

Construction of subclones and DNA hybridizations. λgt11SfI recombinant phage DNA was isolated as described by Silhavy et al. (28). Plasmid subclones of the insert DNAs were prepared in pUC8 by ligating electroeluted insert fragments (removed from λgt11SfI DNA by *EcoRI* digestion and agarose gel electrophoresis) with *EcoRI*-cleaved, phosphatase-treated pUC8 DNA (15). The ligated material was used to transform competent *E. coli* HB101 cells; transformants were selected on L agar supplemented with 50 μg of ampicillin per ml. Recombinants were verified by plasmid analysis of 2-ml overnight cultures (1). Insert DNA from pUC8 recombinant plasmids was labeled with [α - 32 P]dCTP by nick translation (New England Nuclear Corp., Boston, Mass.) and hybridized to *EcoRI*-digested λgt11SfI DNAs and to pWR110 and wild-type M90T invasion plasmid DNAs digested with various combinations of the restriction enzymes *EcoRI*, *BamHI*, *BglII*, *HindIII*, and *PstI*. Southern blot hybridization was used to detect overlapping sequences among the λgt11SfI *ipa* clones and to map the region of the M90T invasion plasmid conferring the invasive phenotype.

RESULTS

Identification of λgt11 clones reactive with rabbit screening antiserum. The λgt11 expression library of pWR110 DNA, constructed as described above, yielded phage titers of 10^6 PFU/ml without amplification. Rabbit antisera to virulent *S. flexneri* M90T, previously absorbed with M90T-A2 cells, Y1090 cells, and λgt11 phage, were used to identify λgt11 recombinants expressing pWR110 antigens (λgt11SfI). Greater than 90% of the packaged phage DNA contained plasmid sequences inserted into the *lacZ EcoRI* cloning site of λgt11, as determined by the proportion of colorless and blue plaques found on X-Gal plates of λgt11SfI-infected Y1090 cells. When reacted with the screening antisera, 8 to 12% of the recombinant phage gave positive signals. Although λgt11SfI plaque size was uniform, the signal produced by the antigen-positive recombinants varied in inten-

sity. Therefore, 30 strong-signal (S) and 10 weak-signal (W) plaques were selected at random for further study. Each λgt11SfI clone was plaque purified three times, the purification being monitored with rabbit screening antisera, and a high-titer phage lysate was prepared.

Antigen expression from Y1089::λgt11SfI lysogens. Expression of antigen genes cloned in λgt11 can be dependent on or independent of *lac* promoter induction (29, 33). The synthesis and translation of a fused transcript, comprising the β-galactosidase gene and a portion of the cloned antigen gene, are characteristic of *lac*-dependent expression. Lysogens that express antigen genes independent of *lac* induction and do not synthesize fusion proteins contain recombinant λgt11 prophage whose insert sequences provide the necessary transcriptional and translation start signals for synthesis of a complete or truncated form of the antigen gene. To determine whether antigen production in the λgt11SfI recombinants was controlled by the *lac* promoter, whole-cell lysates of the 40 Y1089::λgt11SfI lysogens were grown in the presence or absence of IPTG and probed with anti-β-galactosidase and rabbit screening antisera in a Western blot analysis, an example of which is presented in Fig. 1.

Y1089::λgt11SfI lysogens W71, S58, S39, and S26 synthesized an inducible 116-kDa protein reactive only with anti-β-galactosidase (as did Y1089::λgt11), indicating that expression of invasion plasmid antigens in these lysogens was not *lac* dependent (Fig. 1A). Analysis with the rabbit screening antisera showed that lysogens W71 and S39 synthesized *lac*-independent invasion plasmid antigens of approximately 58 kDa. Lysogens S58 and S26 produced *lac*-independent antigens whose sizes did not match any of the known *ipa* gene products (indicated to the right of the M90T positive control lane in Fig. 1B) and therefore represented truncated amino-terminal peptides of one or more of these antigens. In contrast, Y1089::λgt11SfI-S60 synthesized a *lac*-dependent antigen that was larger than 116 kDa; this product reacted with both the anti-β-galactosidase and rabbit screening antisera, indicating a fusion protein of β-galactosidase and an unspecified invasion plasmid antigen. Of the 40 Y1089::λgt11SfI lysogens tested, 8 produced β-galactosidase-antigen fusion peptides, while the lysogens of two IPTG-induci-

TABLE 1. Polypeptide products and insert DNA size of λ gt11Sf *ipa* clones

| λ gt11Sf <i>ipa</i> clone | Polypeptide ^a synthesized by lysogen, size (kDa) | EcoRI-cleaved insert DNA size (bp) |
|--------------------------------------|---|---|
| <i>ipaB</i> | | |
| S6 | NonI, 37 | 1,800 |
| S12 | I, >116 | 375, 350 |
| S19 | NonI, 15 and 25 | 1,300, 395 |
| S43 | NonI, 15 and 25 | 1,300, 730, ^b 420 |
| S50 | NonI, 20 | 500, 400 |
| S58 | NonI, 30 and 25 | 2,400, ^b 500 |
| S60 | I, >116 | 1,000, ^b 470, 300 |
| S8 | NonI, 57; I, >116 | 1,800, 2,150 |
| W2 | ND | 600 |
| <i>ipaC</i> | | |
| S17 | I, >116 | 1,100, 500 |
| S29 | NonI, 43 | 2,000, 1,150 |
| S44 | NonI, 30 and 43 | 1,800, 1,030 |
| W8 | I, >116 | 1,700 |
| W16 | NonI, 30 | 1,800 |
| W18 | NonI, 30 and 40 | 1,850, 750 |
| S45 | ND | 500 |
| W12 | NonI, 30 | 1,500 |
| W22 | NonI, 43 | 1,750 |
| <i>ipaBC</i> | | |
| S47 | I, >116; NonI, 20 | 2,050, 1,450, ^b 1,150 ^b |
| W28 | I, 20 and 40 | 1,200, 1,000 |
| <i>ipaD</i> | | |
| S10 | I, >116 | 870 |
| S26 | NonI, 45 (43 and 39) | 1,250, 600 ^b |
| <i>ipaH</i> | | |
| S63 ^c | NonI, 58 ^c | 3,500, 950 |
| W7 | I, >116 | 950 |
| S54 | I, 58 | 1,100, 1,300 |

^a NonI, Not IPTG inducible (*lac* independent); I, IPTG inducible (*lac* dependent); ND, polypeptide not detected.

^b Insert fragments obtained from the 2.9- or 2.1-MDa cryptic ColE1-derived plasmids of *S. flexneri* serotype 5, as determined by hybridization with ColE1 DNA.

^c The following λ gt11Sf *ipaH* prophage synthesized *lac*-independent 58-kDa antigen as in λ gt11Sf-S63: S16, S25, S31, S39, S53, S52, S66, S67, W20, S40, S42, S46, S48, S49, and W71.

ble clones (S54 and W28) contained operon fusions that resulted in the production of antigens not fused to β -galactosidase. Twenty-eight lysogens synthesized *lac*-independent complete or truncated peptides of the invasion plasmid antigens (Table 1); for two lysogens (S45 and W2) (Table 1), no polypeptide product was detected in the Western blots, a possible indication that radically truncated *ipa* peptides were synthesized by the small inserts (500 to 600 base pairs [bp]) contained in these recombinants. A control Western blot of Y1089:: λ gt11 probed with the rabbit screening antisera revealed no reactive antigens (Fig. 1B).

Identification of *ipa* genes and products in λ gt11Sf recombinants. Western blot analysis of IPTG-induced and uninduced Y1089:: λ gt11Sf lysogens probed with the rabbit screening antisera revealed the type of control governing the expression of the cloned pWR110 antigens but could not be used to identify the *ipa* gene isolated in a given λ gt11Sf recombinant. Therefore, antigen-selected antibodies were prepared from each λ gt11Sf recombinant and used in a Western blot analysis of polypeptides obtained from virulent

M90T cells. Invasion plasmid antigens of the virulent M90T cells that were reactive with the antigen-selected antibodies from a single λ gt11Sf recombinant indicated the polypeptide(s) cloned in that λ gt11Sf recombinant and its corresponding *ipa* gene(s) (Fig. 2). By this criterion, recombinants λ gt11Sf-S17 and λ gt11Sf-S44 contained *ipaC* sequences directing the synthesis of 43-kDa (polypeptide c) epitopes; λ gt11Sf-S12 and λ gt11Sf-S19 were identified as *ipaB* clones (57 kDa; polypeptide b), and λ gt11Sf-S10 was identified as an *ipaD* clone (39 kDa, polypeptide d).

An analysis of the 40 λ gt11Sf clones (Table 1) revealed that 28 separate recombinants bound antibody that reacted with the polypeptide b band and presumably contained the *ipaB* gene. Likewise, eight recombinant phage contained the *ipaC* gene, two contained *ipaD*, and two were *ipaBC* recombinants (i.e., coding for epitopes of both the 57- and 43-kDa antigens). Further studies, presented below, demonstrated that 17 of the 28 presumptive *ipaB* clones produced an antigen that was similar in size to, but immunologically distinct from, polypeptide b. This previously unrecognized 58-kDa peptide was termed polypeptide h, and the corresponding gene was termed *ipaH*. Thus, of the 28 presumptive *ipaB* recombinants, 17 were found to be *ipaH* clones and 11 were true *ipaB* recombinants.

Complete, truncated, and β -galactosidase fusions of the 58-, 57-, 43-, and 39-kDa antigens were found among the λ gt11Sf recombinants examined (Table 1). With two exceptions (λ gt11Sf-S47 and λ gt11Sf-W28), each clone synthesized only one antigen, although the DNA insert size (Table 1) indicated that many of the clones could accommodate the synthesis of more than one polypeptide.

DNA hybridization studies of λ gt11Sf recombinants. Phage DNA extracted from various λ gt11Sf recombinants and

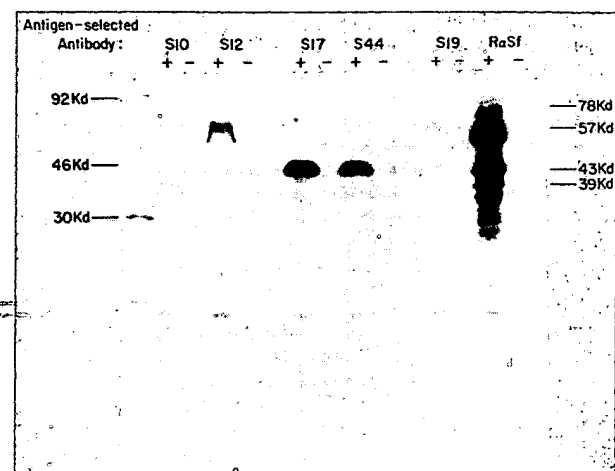


FIG. 2. Western blot of whole-cell lysates of M90T (+) and invasion plasmid cured M90T-A2 (-) probed with antigen-selected antibody and ¹²⁵I-labeled staphylococcal protein A. The λ gt11Sf recombinant from which the selected antibody was derived is indicated by the numbers across the top of the gel. To the left are ¹⁴C-labeled protein standards (molecular masses indicated); the extreme right-hand portion of the blot shows M90T and M90T-A2 lysates probed with the screening rabbit antisera (RaSf). The positions of the 78-, 57-, 43-, and 39-kDa antigens in M90T were used to determine the identity of the antigen reacting with a given selected antibody and hence the identity of the antigen cloned in a particular λ gt11Sf recombinant.

digested with *EcoRI* gave insert DNA sizes of 0.35 to 4.65 kb (Table 1). DNA inserts from several of the λ gt11Sfi clones were isolated and used to hybridize homologous and heterologous λ gt11Sfi DNAs cleaved with *EcoRI* and immobilized on nitrocellulose filters. Hybridization patterns were consistent with the *ipa* gene identifications separately determined for each recombinant phage by using antigen-selected antibody (Table 1; Fig. 2). λ gt11Sfi-*ipaB* recombinants contained insert sequences homologous to each other but not to insert DNA from λ gt11Sfi-*ipaC* or λ gt11Sfi-*ipaD* recombinants. Similarly, consistent hybridization patterns were observed when most *ipaC* or *ipaD* recombinants were used as probes against the bank of λ gt11Sfi recombinants. However, a close linkage of the *ipaC* and *ipaD* loci was inferred, since λ gt11Sfi *ipaD* inserts of recombinants S26 and S10 hybridized selected *ipaC* recombinants that contained large insert DNA fragments (e.g., S29, S44) (see Fig. 5). λ gt11Sfi-*ipaBC* clones S47 and W28, characterized as synthesizing epitopes of both the 57- and 43-kDa invasion plasmid antigens, were found to hybridize both *ipaB* and *ipaC* λ gt11Sfi recombinant DNAs, as expected. A summary of the hybridization data obtained from these experiments is presented below in the genetic map of the *ipaB*, *ipaC*, and *ipaD* loci (see Fig. 5).

The insertion element *IS1* has been found to be a common constituent of the *Shigella* genome (16, 18) and has been implicated as a modulator of the Congo red-binding phenotype, a property indirectly associated with *Shigella* virulence (5, 25). We wanted to determine the number of *IS1* copies on the pWR110 plasmid and to find whether *IS1* sequences were linked with any of the cloned *ipa* genes. An *IS1* probe was prepared by excising an *IS1* internal region from the *EcoRI* H fragment of resistance plasmid NR1 (19, 20). *EcoRI* fragment H was digested with *PstI* and *NcoI*, generating a 618-bp *IS1* probe fragment, 30 bp of which consisted of non-*IS1* DNA. Hybridization to plasmid pWR110 DNA digested with *EcoRI* and *BglIII* (no cut sites in *IS1*) and *PstI*

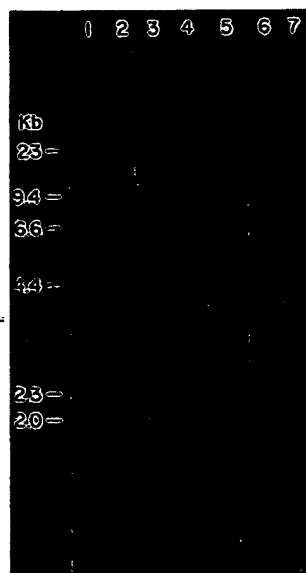


FIG. 3. Hybridization of *IS1* to pWR110 DNA digested with *EcoRI* (lane 1), *BglIII* (lane 2), *BglIII-EcoRI* (lane 3), *PstI* (lane 4), and *PstI-EcoRI* (lane 5). Lanes 6 (*EcoRI*-cut pRR134) (22) and 7 (*HindIII*-cut λ DNA) are positive and negative controls, respectively. The preparation of the 618-bp *IS1* probe is described in the text.

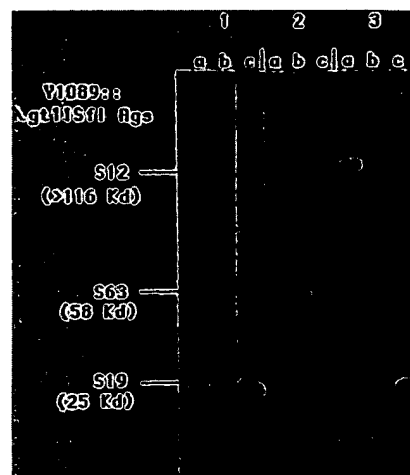


FIG. 4. Western blot analysis of Y1089:: λ gt11Sfi *ipaH* and *ipaB* lysogens probed with antigen-selected antibodies prepared from recombinants S12 and S19 (*ipaB*) and from S63 (*ipaH*). Antigen-selected antibodies were reacted with IPTG-induced lysogens of homologous or heterologous recombinants. In each panel, the arrangement of lysogen lysates is S12 (a), S63 (b), and S19 (c); antigen-selected antibodies prepared from λ gt11Sfi-S19 (panel 1), S63 (panel 2), and S12 (panel 3) were reacted with each lysogen, and positive reactions were interpreted as indicating epitope homology.

(one cut site in *IS1*) revealed the presence of two *IS1* copies on the plasmid DNA (Fig. 3). However, hybridization of this probe to the bank of 40 λ gt11Sfi recombinants did not reveal *IS1* in any of the clones. Thus, the two *IS1* sequences present on pWR110 are not closely linked to *ipa* genes isolated in this investigation.

Identification of a new invasion plasmid antigen, *ipaH*. λ gt11 recombinants directing the *lac*-independent synthesis of a 58-kDa antigen were isolated more frequently than λ gt11Sfi-*ipaC* and λ gt11Sfi-*ipaD* clones (Table 1). Initial identification by the antigen-selected antibody technique suggested that 28 clones were *ipaB* recombinants. However, DNA from 17 of these clones did not hybridize with the remaining 11 λ gt11Sfi-*ipaB* DNAs encoding the synthesis of truncated or β -galactosidase fusion versions of the 57-kDa antigen. Thus it appeared that 17 λ gt11Sfi recombinants directed the synthesis of an antigen similar in size to the *ipaB* product but distinct as determined by DNA sequence homology. The gene encoding this newly defined antigen was called *ipaH*.

To determine whether the *ipaH* and *ipaB* antigens were immunologically related, antigen-selected antibody made from *ipaH* clones (e.g., λ gt11Sfi-S63, λ gt11Sfi-S52, or λ gt11Sfi-S39, which encode the 58-kDa antigen) was reacted with β -galactosidase fusion or truncated *ipaB* antigen (e.g., λ gt11Sfi-S12, λ gt11Sfi-S19, or λ gt11Sfi-S43) in a Western blot of selected Y1089:: λ gt11Sfi lysogens; no cross-reaction was found (Fig. 4). Similarly, antigen-selected antibody made from λ gt11Sfi *ipaB* clones did not react with lysates of Y1089:: λ gt11Sfi *ipaH* lysogens. These data indicate that the *ipaB* and *ipaH* peptides are unrelated immunologically.

A comprehensive analysis of the 40 λ gt11Sfi recombinants showed that antigen-selected antibody prepared from *ipaB*, *ipaH*, *ipaC*, and *ipaD* λ gt11Sfi recombinants did not cross-react with heterologous Y1089:: λ gt11Sfi lysogens, indicating that these antigens are immunologically unrelated and rep-

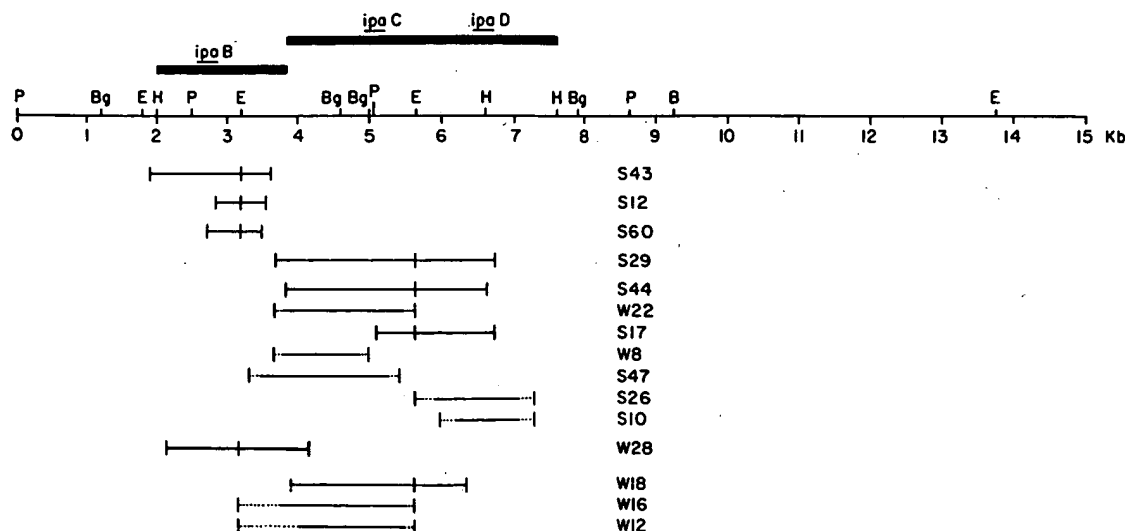


FIG. 5. Genetic map of the *ipaBCD* gene cluster. M90T invasion plasmid DNA was digested with *EcoRI* (E), *BglII* (Bg), *BamHI* (B), *HindIII* (H), and *PstI* (P), singly or in combination, and probed with purified insert DNA from various pUC8 subclones of λ gt11Sfi recombinants (indicated by bars below the genetic map). In addition, the complete bank of λ gt11Sfi recombinants was hybridized with each insert fragment tested, allowing confirmation on the positioning of insert fragments relative to the invasion plasmid DNA and to each other. The exact position of insert fragments that do not contain *EcoRI*-cut sites may vary as indicated by the dotted lines. The maximal boundaries of each *ipa* gene are indicated with solid bars above the restriction map.

resent distinct outer membrane protein antigens (unpublished observations).

Construction of a genetic map of the *ipa* genes. Insert fragment DNAs from several λ gt11Sfi recombinants were subcloned into pUC8. Radiolabeled insert DNA was hybridized to M90T invasion plasmid DNA digested with combinations of five restriction enzymes (*EcoRI*, *BamHI*, *BglII*, *HindIII*, and *PstI*) and immobilized on nitrocellulose filters. Consideration of λ gt11Sfi insert fragment lengths (Table 1) and the size of invasion plasmid restriction fragments hybridized by the probes allowed us to construct a detailed map of the *ipaB*, *ipaC*, and *ipaD* genes (Fig. 5). Positioning of all λ gt11Sfi insert fragments relative to the invasion plasmid restriction map was confirmed by DNA cross-hybridization between different λ gt11Sfi insert fragments, as described above. On the basis of an analysis of Y1089:: λ gt11Sfi protein products and antigen-selected antibody data (Table 1), the *ipaB* gene was localized to a 2.0-kb DNA segment that lies immediately adjacent to a 3.9-kb region containing the *ipaC* and *ipaD* loci. The close linkage of the *ipaB* and *ipaC* genes was further verified by the finding that insert DNA from recombinants λ gt11Sfi-S47 and λ gt11Sfi-W28 (which encode epitopes of both the 57- and 43-kDa antigens) overlapped the *ipaB* and *ipaC* regions (Fig. 5).

Three λ gt11Sfi *ipaH* clones (S52, S63, and W7) were used in similar mapping experiments. Insert DNA from these recombinant molecules hybridized strongly to DNA isolated from all 17 *ipaH* recombinants described in Table 1. However, insert DNA probes S52, S63, and W7 did not hybridize λ gt11Sfi *ipaB*, *ipaC*, *ipaD* recombinants. Hybridization of these three probe DNAs with endonuclease-digested M90T invasion plasmid DNA gave a distinct pattern of bands that did not correspond to the sequence of restriction enzyme cut sites found in the *ipaBCD* region of the invasion plasmid (data not shown). These data indicate that the *ipaH* locus is spatially separated from the *ipaBCD* gene cluster on the M90T invasion plasmid.

DISCUSSION

This report presents the molecular cloning and characterization of four *S. flexneri* serotype 5 invasion plasmid antigen (*ipa*) genes and their products by using the λ gt11 expression vector. Rabbit antisera, specific for M90T invasion plasmid antigens b (57 kDa, *ipaB*), c (43 kDa, *ipaC*), d (39 kDa, *ipaD*), and h (58 kDa, *ipaH*), were used to detect λ gt11 recombinants that encoded the synthesis of various epitopes of these antigens. A survey of the 40 λ gt11Sfi recombinants and their corresponding lysogens, by using Western blot analysis and antigen-selected antibodies, indicated that complete, truncated, and β -galactosidase fusions of genes *ipaB*, *ipaC*, *ipaD*, and *ipaH* had been isolated (Table 1). In addition, insert DNAs from the λ gt11Sfi recombinants were used in hybridization experiments with M90T invasion plasmid DNA to construct a genetic map of the *ipa* gene region. This map demonstrates that there is a close linkage of genes *ipaB*, *ipaC*, and *ipaD* while gene *ipaH* does not map to the same region (Fig. 5).

Although the majority of λ gt11Sfi recombinants characterized in this work encoded the synthesis of only one *ipa* gene product, two recombinants, λ gt11Sfi-S47 and λ gt11Sfi-W28, were found to synthesize epitopes of both the *ipaB* and *ipaC* polypeptides. Use of antigen-selected antibody prepared from heterologous *ipaB* and *ipaC* clones demonstrated that the fusion protein in Y1089:: λ gt11Sfi-S47 is an *ipaB* fusion; the smaller, *lac*-independent 20-kDa protein was not detected in the lysogen but was found in a pUC8 subclone of the λ gt11Sfi-S47 insert probed with a monoclonal antibody specific for the 43-kDa *ipaC* protein (data not shown). The 40- and 20-kDa antigens synthesized by Y1089:: λ gt11Sfi-W28 reacted with *ipaB* monoclonal and antigen-selected antibody and *ipaC* monoclonal and antigen-selected antibody, respectively. When insert DNAs from clones S47 and W28 were mapped with respect to the parental plasmid it was found that both clones overlapped the *ipaB*/*ipaC* bound-

ary (Fig. 5) and could account for the synthesis of epitopes from both antigens. A clone encoding the synthesis of both *ipaC* and *ipaD* epitopes has not been identified, despite the close linkage of these genes and the isolation of λ gt11Sfl recombinants that span the *ipaC*/*ipaD* boundary (e.g., S26 and S44). Although lysogens such as Y1089:: λ gt11Sfl-S26 and Y1089:: λ gt11Sfl-S44 synthesize more than one polypeptide, as determined in Western blots with the rabbit screening antisera (Fig. 1; Table 1), the use of antigen-selected antibodies prepared from these clones and reacted with M90T invasion plasmid proteins indicated that only one *ipa* gene had been cloned. This finding suggests that nonimmunogenic portions of the *ipaC* and *ipaD* polypeptides have been cloned in λ gt11Sfl-S26 and λ gt11Sfl-S44, respectively, or that the additional polypeptides in such clones represent specific degradation products of a single cloned antigen. The latter possibility is supported by the noted lability of these outer membrane proteins (7, 8) and by the detection of multiple peptides in Y1089:: λ gt11Sfl lysogens carrying insert DNA that maps entirely within a defined *ipa* gene (e.g., S19, S43, and W18; Fig. 5).

Understanding the regulation of *ipa* gene expression might provide an important insight into how the production of the invasive phenotype is coordinated with expression of other virulence determinants in *S. flexneri*. The production of invasion plasmid antigens is known to be temperature regulated (as is the expression of virulence genes in other enteropathogenic bacteria) such that synthesis of the antigens is repressed below 37°C (13, 14). An analysis of the types of *ipa* gene recombinants obtained in the λ gt11Sfl library and the expression of *ipa* genes in Y1089:: λ gt11Sfl lysogens and HB101 (pUC8 *ipa*) subclones suggests two models to account for temperature-regulated *ipa* gene expression. In the first model, the various *ipa* genes (or gene clusters) constitute individual units of transcription making up a larger, temperature-responsive regulon. Coordinate expression of the genes is affected by a positive activator protein that allows transcription of each gene in response to the temperature of the environment. A temperature-controlled regulon of this sort has previously been described for the low calcium response of *Yersinia pestis* (31). Results of experiments with defined *ipaB*, *ipaC*, and *ipaD* fragments hybridized to total RNA isolated from virulent *S. flexneri* indicate the presence of 3.6- and 1.6-kb transcripts synthesized from the *ipaBCD* region (J. M. Buysse, M. Venkatesan, C. K. Stover, E. V. Oaks, and D. J. Kopecko, Abstr. XIV Int. Congr. Microbiol., P.I9-27, p. 221, 1986). In addition, selected λ gt11Sfl recombinants expressing complete and truncated 57- or 43-kDa antigens were subcloned into plasmid pUC8, and protein expression of the HB101(pUC8 *ipa*) subclones was analyzed by Western blot analysis with rabbit screening antisera and monoclonal antibodies to the *ipaB* and *ipaC* proteins. Each of the subclones was found to synthesize a peptide identical in size to the one produced by the parental Y1089:: λ gt11Sfl lysogen (data not shown). This control experiment demonstrated that the *lac*-independent production of *ipaB* and *ipaC* antigens in Y1089:: λ gt11Sfl lysogens is controlled by endogenous promoters carried on the pWR110 insert DNA and does not result from λ -directed transcription, particularly that directed by the λ *lom* promoter which lies at the 3' end of the λ gt11 *lacZ* gene and can, with the proper orientation of insert DNA, direct the synthesis of a *lac*-independent fusion protein (4). Taken together, these observations indicate that the *ipaBCD* gene cluster encodes discrete transcriptional units, compatible with the proposed regulon model.

The alternative operon model postulates a single polycistronic unit encoding the *ipa* genes that is subject to coordinate transcriptional regulation via a repressor molecule. The repressor responds in turn to some external signal, most likely the temperature of the growth environment. The operon model is supported by the observed clustering of Tn5 insertions that block the invasive phenotype of cosmid clone pHS4108 (13) and by the demonstrated linkage of genes *ipaB*, *ipaC*, and *ipaD*. However, it is not supported by data gathered on the expression of *ipa* genes in Y1089:: λ gt11Sfl lysogens and HB101(pUC8) subclones or by the observed physical separation of *ipaH* from *ipaBCD*. If the operon model were correct, one would also expect a bias in the types of *ipa* gene expression observed, dependent on the position of a particular *ipa* gene within the operon. The initial gene of the operon would be represented in the λ gt11 expression library as a complete, truncated, or β -galactosidase fusion polypeptide, depending on whether the transcription start signal is provided by the insert DNA or the *lacZ* gene of the λ gt11 vector. Genes located downstream of the 5'-proximal polypeptide gene, however, would be present in the library only as β -galactosidase fusion peptides or as complete (or amino-terminal peptide) antigens synthesized in conjunction with the initial gene of the operon. This hypothesized bias was not found for the types of *ipa* clones isolated (Table 1). In fact, the isolation of individual β -galactosidase fusion, truncated, and complete antigens for each *ipa* gene implies that the genes are encoded on separate transcriptional units.

An intriguing result of the analysis of the 40 Y1089:: λ gt11Sfl lysogens was that 40% of the recombinants (16 of 40) synthesized a *lac*-independent 58-kDa antigen designated polypeptide h (*ipaH*). Initially, these clones were thought to be λ gt11Sfl-*ipaB* recombinants, since antigen-selected antibody prepared from them appeared to react with the 57-kDa antigen found in virulent M90T. However, insert DNA isolated from these putative "*ipaB*" clones did not hybridize λ gt11Sfl-*ipaB* DNA from clones encoding the synthesis of truncated or β -galactosidase fusions of the 57-kDa antigen. Furthermore, antibody selected from λ gt11Sfl *ipaH* clones did not react with Y1089:: λ gt11Sfl *ipaB* lysogens and vice versa (Fig. 4), leading us to conclude that genes *ipaB* and *ipaH* produce antigenically distinct proteins of similar molecular weight that are not resolved on one-dimensional SDS-PAGE Western blot analysis. The *ipaH* locus is spatially separated from the *ipaBCD* gene cluster, since *ipaH* probes did not hybridize λ gt11Sfl *ipaBCD* DNA and the *ipaH* restriction endonuclease map was distinct from that of the *ipaB*, *ipaC*, and *ipaD* loci (manuscript in preparation). The separation of *ipaH* from other *ipa* genes in *S. flexneri* is analogous to the separation of the *virF* and *virG* genes on the *S. flexneri* 2a invasion plasmid (pMYSH6000) from a 33-kb region of pMYSH6000 controlling several phenotypes associated with *Shigella* virulence (26).

The restriction map of the pWR110 *ipaBCD* region resembles that constructed for a cosmid clone (pHS4108) of the wild-type *S. flexneri* serotype 5 invasion plasmid that restores HeLa cell invasiveness to plasmid-cured avirulent *S. flexneri* cells (13). Two regions on the 37-kb segment cloned in pHS4108 have been defined by Tn5 mutagenesis as essential for the invasive phenotype. One cluster of five Tn5 insertions spans the ends of two large *EcoRI* fragments (11.5 and 17 kb) that are separated by an intervening 1.5-kb *EcoRI* fragment. A 7.6-kb *EcoRI* fragment flanked by two smaller *EcoRI* fragments defines the second region, which is removed by some 8 to 9 kb from the end of the 11.5-kb *EcoRI*

fragment. Purified insert DNA from *ipaB*, *ipaC*, and *ipaD* did not hybridize the 11.5 or 17.0-kb *EcoRI* fragments of the M90T invasion plasmid (data not shown); however, selected *ipaC* and *ipaD* probes did hybridize an 8.0-kb *EcoRI* fragment, while other *ipaC* and all of the *ipaB* probes hybridized two flanking and contiguous 2.3- and 1.4-kb *EcoRI* fragments. Although we have not hybridized our *ipaBCD* clones to cosmid pHS4108 DNA, it seems reasonable to assume that the 8.0-kb *EcoRI* fragment and two smaller flanking *EcoRI* pieces defined here (Fig. 5) correspond to the 7.6-kb *EcoRI* region of pHS4108, particularly since cosmid clone pHS4108 synthesizes the 57-kDa (*ipaB*), 43-kDa (*ipaC*), and 39-kDa (*ipaD*) antigens (13).

It has recently been shown by Watanabe and Nakamura (30) that a molecule containing contiguous 2.6- and 4.1-kb *HindIII* fragments, derived from the *S. sonnei* 120-MDa invasion plasmid and cloned into pACYC184, can complement *TnI* insertions in the invasion plasmid that eliminate the invasive phenotype. The 4.1-kb *HindIII* fragment was found to hybridize with a similar-sized *HindIII* fragment from invasion plasmids of enteroinvasive *E. coli*, *S. boydii*, *S. dysenteriae*, and *S. flexneri*. In our study, we have located the *ipaBCD* genes of *S. flexneri* serotype 5 to contiguous 1.0- and 4.6-kb *HindIII* fragments. We have also found that probes of the *ipaB*, *ipaC*, and *ipaD* loci specifically hybridize plasmid DNA from a number of virulent dysenteric bacilli (manuscript in preparation). The relationship, if any, of the 1.0- and 4.6-kb *HindIII* fragments described here to the fragments isolated from *S. sonnei* is unclear at present.

In contrast to the situation found for *Y. pseudotuberculosis*, in which a single, cloned genetic locus imparts to an *E. coli* K-12 recipient the ability to invade cultured epithelial cells (9), *Shigella* spp. and enteroinvasive *E. coli* rely on multiple genetic determinants to accomplish the same end (10, 21). Owing to the relative complexity of the *Shigella* invasion system, dissection of invasion genes with λ gt11 should facilitate attempts to identify and isolate genes involved in epithelial cell invasion. The λ gt11 cloning system can be used to study *ipa* genes outside of the milieu of surrounding DNA that may not be directly responsible for the invasive phenotype. The λ gt11 vector also provides a way of defining new plasmid antigens that would not be detected by conventional SDS-PAGE analysis of wild-type or cosmid cloned invasion plasmid proteins, as demonstrated by the identification of *ipaH* in this work. The analysis of a large number of λ gt11Sf1 *ipa* recombinants by using antigen-selected antibody to probe homologous and heterologous Y1089:: λ gt11Sf1 lysogen products has been used to define individual epitopes of the 57- and 43-kDa antigens (manuscript in preparation). Future efforts will be directed at determining the relative contributions of each *ipa* gene to the invasive phenotype and at assessing their potential as subunit vaccines.

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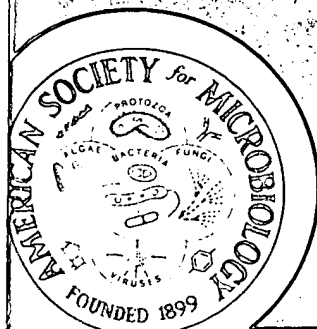
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